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THE DIAGNOSIS OF MOUSE POX (ECTROMELIA)

BY MEANS OF AGAR-GEL SOLUTION

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Mouse pox virus infection is easily recognizeable when the classical syndrome which leads to the characteristic form of ectromelia is present (1). However, this pathognostic picture of the cutaneous lesion and spontaneous amputation is not always indicative. Laboratory methods, which either show the virus or its antibody, must be used for the diagnosis of the acutely lethal as well as the inapparent infection.

Isolation of the virus in the mouse, in embryonic chicken eggs or in tissue culture requires 2 to 4 days and can only be demonstrated in combination with a neutralization test. Briody (2) introduced the rapid diagnostic method of demonstrating the antibody in serum of infected mice which inhibits hemagglutination. This is, however, also usable after standardization for a microtitration system (3).

The agar-gel precipitation method of Ouchterlony (4) is generally the simplest serologic technique for the diagnosis of several animal viral infections (5, 6, reviewed in 7); studies on viruses of the pox group have also been reported (8,9).

In these experiments we demonstrate the antibody as well as the antigen in mice experimentally infected with ectromelia using the precipitation reaction.

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Material and Methods

Virus: The neurovaccine strain Levaditi and the ectromelia strain Elberfeld were used as antigens as well as the Mannheim, Freiburg and Munich strains isolated from embedded materials. According to the technique of Westwood et al., inoculated chicken eggs, preincubated for 10 days, were opened after 72 hours, the generalized chorioallantois membranes (CAM) were harvested and washed in isotonic sodium chloride solution (10). They were diluted 1:2 (wet weight: volume) with this solution and homogenized (Ultra-Turrax, Jahnke and Kunkel Company). After centrifugation (1000g, 15 min) the supernatent was taken as antigen.

Antiserum: We used the same mouse ectromelia hyperimmune serum as for inhibiting the hemagglutination reaction (3).

Agar-gel precipitation: According to Crowle's procedure (11) purified agar (Difco Special Agar Noble) was placed in desalinated water in a 2% suspension and dissolved by heating in a water bath. For better adhesion of the agar-gel to the slides, the latter were prepared by taking each through a 0.5% agar solution in desalinated water and drying in a warming cabinet.

Sodium chloride was dissolved in phosphate buffer (ionic strength 0.15, pH 7.4) in an amount which corresponded to twice that required for isotonisity (1.6g/1000 ml). The solution was heated to 37° C, mixed in equal portions with the agar solution and poured immediately. Agents which inhibit germination were not added.

3.0 ml of the agar medium was pipetted onto the prepared slides.

In previous experiments we had determined the most favorable bore diameter and preparation, reaction temperature and time per optimal read-out.

Holes of 3 mm. diameter, 5 mm. apart, were punched out in the pattern of an equilateral triangle. We transferred the liquid reagents with a glass capillary, organ pieces being packed in as densely as possible.

The reactions were recorded photographically. The gel was removed from the slide with a 1% glycerine solution, transferred with a buffered sodium chloride solution to a flat petri dish and used as the negative in an enlarger.

Experimental

1. Demonstration of precipitating antibodies

Gispen's (9) studies showed that ectromelia antigen could be precipitated by vaccine antiserum using Oudin's agar-gel technique (12). According to these results we could expect that sera of pox-sick mice would also precipitate ectromelia- or vaccine-antigen. For the purpose of finding a simple diagnostic method we studied various antigens using Ouchterlony's technique (4).

a) Test antigens from infected fetal membranes:

Both the vaccine and the ectromelia-antigen gave a precipitation against ectromelia antiserum from mice. A band formed after 24 hours which was followed by another in the vaccine-antigen after 48 hours near the serum spot.

CAM-antigens are also suitable for demonstration of the antibodies in infected mice. In order to confirm the specificity of the reaction an empty serum was put on one side of the "patients'" serum and an antiserum on the other side. Small amounts of antibody, not demonstrable alone, arose as a continuation of the bands towards the antiserum, a phenomenon which has been described as a strengthening or "recruiting" effect (5,20).

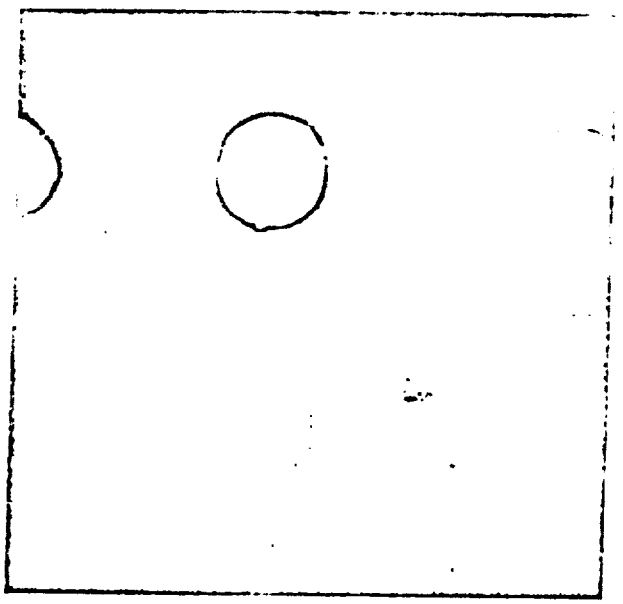

Figure 1. Demonstration of precipitating ectromelia antigens in organ material, liver (above), spleen (below). Black circles: mouse ectromelia antiserum.

Table 1. Demonstration of precipitating antibodies in the sera of mice orally infected with ectromelia

(a) Tage post infectionem	(b) Virusdosis			
	10^{8}	10^{7}	10^{6}	10^{6} LD_{50}-Einheiten (c)
5			± (0)*	
7			± (0)	
9			± (0)	
10	+ (160)**			
11	+ (320)			
12	+ (320)			
20		+ (320)		
25			+ (160)	
30			+ 160)	
32		+ (160)		
55				+ (40)
90		+ (80)		
95			+ (320)	

* ± gel turbidity ** + precipitation bands
( ): reciprocal liter of hemagglutination inhibition

[Legend]: a) Days after infection; b) virus dose; c) LD_{50}-units.

Table 2. Demonstration of precipitating antibodies, after intraperitoneal challenge-infection with 10^{6} LD_{50}-units of ectromelia virus

(a) Oral Ektromelie-infizierte Mäuse		(c) Tage nach Belastung	(d) Mit Vakzinevirus geimpfte Mäuse		
(b) Ausgangswerte			(e) Ausgangswerte		
−(40)*	−(0)		±(0)**	−(0)	−(0)
+ (160)†		4			
		5	+ (80)		
	+ (320)	6		+ (160)	
		9			+ (320)

* − no precipitation ** ± gel turbidity
† + precipitation bands
( ): reciprocal liter of hemagglutination-inhibition

[Legend]: a) mice orally infected with ectromelia; b) initial values; c) days after loading; d) mice inoculated with vaccine virus; e) initial values.

Test antigens from infected mice: The livers and spleens from mice which had died from an ectromelia infection were tested against ectromelia antiserum. The majority of organs showed precipitation which occurred in several (maximal six) discernible bands (Figure 1). In the following we focused attention on the organ-antigens, running blanks and antiserum in all tests as controls.

b) Demonstration in serum:

For clarification of the question whether precipitating antibodies are demonstrable, mice (NMRI-female, 18 to 22 g) were infected with ectromelia virus in doses between 10^1 and 10^6 LD50-units via the intraperitoneal (i.p.), intraplantar (i.pl.) and oral route. In no case could antibodies be demonstrated with certainty before the 10th post-infection day (d.p.i.).

Table 1 shows the results of studies on orally infected groups.

In mice which were apparently not infected orally (13) are loaded with 10^6 LD50-units of ectromelia virus intraperitoneally, precipitating antibodies become apparent on 4 d.p.i. The same reduction in the interval was observed when animals which had not been protectively inoculated were infected with vaccine virus (14) (Table 2).

II. Evidence of Precipitating Antigens

The regularity with which precipitating antigens were demonstrable in the livers and spleens of mice which had succumbed to ectromelia led us to assume that this should also hold true for other organs. Therefore, we systematically studied parts of the digestive and urogenital tracts, the endocrine and reticulo-endothelial system as well as heart, lungs, brain, serum and when indicated the cutaneous lesion in agar-gel precipitation. The mode of infection and doses were chosen so that the disease occurred in the three main forms: the acute - after i.p. infection-, the classic, chronic - after i.pl. infection - and the inapparent - after oral infection.

a) Demonstration in organs of dead animals:

As is apparent from Table 3, precipitating antigen was demonstrable in the majority of organs studied. We routinely found soluble antigen in organs of the reticulo-endothelial system, after i.p. as well as after oral infection of mice. Precipitating antigens were demonstrable almost as frequently in the small and large intestine and in the kidney and bladder. Skin tests in the region of the

Table 3. Demonstration of precipitable antigens in organs of mice infected with Coxsackia and which subsequently died

Dünndarm 1)	Niere 2)	Haut (Plantar) 3)	Leber 4)	Milz 5)	Lymph II	Lunge 6)	Pankreas 7)	Gehirn 8)	d.p.i.	Herz 9)	Magen 10)	Dickdarm 11)	Blase 12)	Ovar 13)	Thymus	Nebenniere 14)	Knochenmark 15)	Lymph I	Lymph III
a) intraperitoneale Infektion																			
+	+	−*	+	+	+	±**	+***	−	4										
+	+	−	+	+	+	±	+	−	5	−	−	+	+	+	+	+	+	+	+
+	+	−	+	+	+	+	+	−	6	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	−	7	−	±	+	+	+	+	+	−	−	+
+	+	±	+	+	+	−	−	−	8										
+	+	±	+	+	±	−	−	−	9										
+	+	−	+	+	−	−	−	−	10	−	+	+	+	+	−	−	−	−	−
b) intraplantare Infektion																			
+	+	+	+	+	+	−	+	−	6										
+	±	−	+	+	±	−	−	−	7										
+	−	+	−	+	−	−	−	−	8										
+	−	+	−	+	−	−	−	−	9										
−	−	+	−	−	−	−	−	−	10										
c) orale Infektion																			
+	+	−	+	+	+	+	+	−	8†										
+	+	−	+	+	+	−	−	−	18	−	±	+	+	+	+	+	+	+	+
−	−	−	−	+	−	−	−	−	22	−	−	+	−	−	−	−	+	+	+
+	+	−	+	+	+	−	−	−	25	+	+	+	+	+	+	+	+	+	+
+	+	−	+	+	+	−	−	−	32	−	+	+	+	+	+	−	+	+	+
−	−	−	+	+	+	−	−	−	36	−	−	+	±	+	+	+	−	+	+

Lymph I: Axillary and popliteal space lymph nodes
Lymph II: Esophageal, cranial, caudal, superficial neck lymph nodes
Lymph III: Inguinal, iliac and mesonteric lymph nodes

* − no precipitation ** ± gel turbidity
*** + precipitation bands
† The times given refer to the deaths collectively after oral infection of individual animals with small doses of virus.

[Legend]: a) intraperitoneal infection; b) intraplantar infection; c) oral infection; 1) small intestine; 2) kidneys; 3) skin (plantar); 4) liver; 5) spleen; 6) lungs; 7) pancreas; 8) brain; 9) heart; 10) stomach; 11) colon; 12) bladder; 13) ovary; 14) adrenals; 15) bone marrow.

Table 4. Demonstration of precipitable antigens in mice infected with ectromelia and then sacrificed

Dünndarm 1)	Niere 2)	Haut (Planto)3)	Leber 4)	Milz 5)	Lymph II	Lunge 6)	Pankreas 7)	Gehirn 8)	d.p.i.	Herz 9)	Magen 10)	Dickdarm 11)	Blase 12)	Ovar 13)	Thymus	Nebenniere 14)	Knochenmark15)	Lymph I	Lymph III	Serum
a) intraperitoneale Infektion																				
±	**	–	*	–	+	***	+	+	–	+	–	3								
+	+	–	+		+		+	+	–	+	–	4								+ (0)
+	+	±	+		+		+	+	–	+	–	5								+ (0)
+	+	+	+		+		+	+	–	+	–	6	+	–	+	+	–	–	–	–	+ (0)
+	+	–	+		+		–	–	±	–	7		–	–	+	+	–	–	–	–	+ (0)
+	+	–	+		+		–	–	±	–	8		–	–	+	+	–	–	–	–	+ (0)
+	–	–	±		+		–	–	–	–	9										
+	+	–	+		–		–	+	–	10		–	+	+	+	+	–	–	+	–	
–	–	–	+		+		–			13		–	–	+	–	–	–	–	+	+	+	
b) intraplantare Infektion																				
±	–	+	+		+		+	–	–	–	4										
–	–	+	–		–		–	–	–	–	5										
–	–	+	+		–		–	–	–	–	6										
+	–	+	–		–		–	–	–	–	7										± (20)
+	+	+	+		+		+	–	–	–	8										
+	–	+	+		+		+	–	–	–	9										+ (40)
–	±	+	–		–		+	–	–	–	10										
–	–	+	–		–		–	–	–	–	11										
c) orale Infektion																				
–	–	–	±		+		–	–	–	–	6†										
+	±	–	+		+		–	–	–	–	7										+ (40)
					–						8–12										
–	–	–	–		+		–	–	–	–	13	–	–	–	–	+	–	–	–	+	–	
–	–	–	+		–		–	–	–	–	16	–	–	–	–	–	–	–	–	+	±	+ (0)
–	–	–	+		–		+	–	–	–	17	+	–	–	–	–	–	–	–	–	–	
+	+	–	+		+		–	–	–	–	20	+	–	+	+	–	–	±	+	+	–	
+	–	–	+		+		+	–	–	–	25	+	+	+	–	+	+	–	+	+	+	

( ): reciprocal titer of hemagglutination inhibition

[For Legend see table 3.]

primary effect regularly gave a positive reaction only after i.p. infection. Brain tissue always gave a negative finding as did lung, heart and stomach.

b) Demonstration in organs of sacrificed animals:

The precipitation results are comparable with those obtained in organs of succumbed animals. The results obtained in orally infected animals are an exception (Table 4).

c) Demonstration in serum:

Serum drawn between 4 and 8 d.p., after i.p. infection routinely precipitates antigens with a simultaneous negative hemagglutination inhibiting reaction. (Table 4)

Discussion

We have been able to routinely demonstrate precipitable proteins which occur during ectromelia infection in the mouse, using the agar-gel technique. Nicoli and Jolibois recently defined the soluble antigens of the pox virus as proteins which could not be sedimented by ultracentrifugation but could be diffused through agar-gel (15). Mayr listed several characteristics which are common to all the pox-S-antigens which he studied; they are not infections, not immunogen, but effective antigens, relatively stable against environmental influences and give a virus specific complement finding and precipitation reaction (16). S-antigen is always found at the site of virus proliferation; it increases there together with the hemagglutinin and the infectiousness. Metcalf and Maitland and Tobin were able to show with the vaccine virus that S-antigens are demonstrable before the mature virus particles and that they are incorporated into the latter (17,18). According to the observations of Downie et al. they only occur in blood in variola infection when the virus proliferation during incubation has been especially intensive (19).

S-antigen was studied in ectromelia virus using the complement binding reaction, and also with agar-gel diffusion according to Oudin's technique (16,9).

We attempted to apply the simple gel precipitation method of Ouchterlony to the diagnosis of ectromelia. At first we used the CAM-antigen, subsequently using organs infected with ectromelia and later turning to succumbed mice.

Precipitating antibodies could routinely be demonstrated in the serum of infected animals from 10 d.p.i., but after oral infection

only when the applied dose of virus was not so small that the infection was not apparent. This time is in good agreement with data for chicken pox (6), other viral diseases of fowl (5), the Blue-Tongue virus (20) etc. Antibodies inhibiting hemagglutination were already formed 3 d.p.i. after intraplantar infection with ectromelia virus (14).

The chief area of application of the procedure for diagnosis, however, must be the antigen demonstration. As we were able to show, liver and spleen, intestine, kidney and, under certain conditions, the cutaneous lesion contain the precipitating antigen for a long time-span. In addition, it can be found in the serum of infected mice between 4 and 9 d.p.i. (Table 4). After intraperitoneal infection the planter skin and the lungs contain S-antigen only when the animals succumb 6 to 7 d.p.i. (or 8, after oral infection). S-antigen is not found in brain.

According to Fenner, the chronic form of the natural disease is imitated soonest by an intraplantar infection (21). Between 3 and 4 d.p.i. an intensive viral proliferation occurs in liver and spleen. We also find precipitating antigens in these organs at this stage. It is interesting that this was demonstrable in the intestine between the 7 and 9 d.p.i., which is the interval in which the infection is normally visible clinically. The intestines of succumbed mice almost always contain S-antigen.

Along with the results of our studies on the oral inapparent infection and the isolation and content of virus in intestine (13), we should like to point out with our precipitation findings the significance of the gastro-intestinal tract for the pathogenesis of mouse pox which has long been underestimated (2,22). The schema presented by Fenner should be extended to include the fact that the virus, after the secondary viremia (6 d.p.i.), is extracted, proliferates and forms a focal lesion not only in the skin but also in the intestine. This would be further explanation for the finding that after i.p. and i.pl. infection of mice with small doses of virus an inapparent "intestinal-carrier" condition can result which possibly stimulates excretion via the gastro-intestinal tract (23).

Summary

The agar diffusion technique of Ouchterlony allows the demonstration of precipitating antibodies in mice from the tenth day onward after infection with ectromelia virus. On the other hand precipitating antigen could regularly be demonstrated in organs of succumbed animals. The Ouchterlony technique is therefore suitable for routine diagnosis of mouse-pox.

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